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HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease)

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Autosomal recessive severe congenital neutropenia (SCN)[1] constitutes a primary immunodeficiency syndrome associated with increased apoptosis in myeloid cells[2,3], yet the underlying genetic defect remains unknown. Using a positional cloning approach and candidate gene evaluation, we identified a recurrent homozygous germline mutation in *HAX1* in three pedigrees. After further molecular screening of individuals with SCN, we identified 19 additional affected individuals with homozygous *HAX1* mutations, including three belonging to the original pedigree described by Kostmann[1]. *HAX1* encodes the mitochondrial protein HAX1, which has been assigned functions in signal transduction[4] and cytoskeletal control[5,6]. Here, we show that HAX1 is critical for maintaining the inner mitochondrial membrane potential and protecting against apoptosis in myeloid cells. Our findings suggest that HAX1 is a major regulator of myeloid homeostasis and underline the significance of genetic control of apoptosis in neutrophil development.

Individuals with autosomal recessive SCN show a paucity of mature neutrophils in peripheral blood and bone marrow and develop life-threatening bacterial infections[7]. SCN constitutes a heterogeneous group of diseases: about 60% of affected individuals of European and Middle Eastern ancestry have dominant heterozygous mutations in the gene encoding neutrophil elastase (*ELA2*)[7,8]. However, the genes mutated in the 'classical' form of SCN, characterized by autosomal recessive mode of inheritance, have remained unknown since the publication of Kostmann's seminal paper[1] 50 years ago. To define the molecular etiology of autosomal recessive SCN, we initiated a genome-wide linkage scan in three unrelated Kurdish families (**Fig. 1**).

All four affected individuals in the index families suffered from recurrent infections due to neutropenia characterized by a maturation arrest at the promyelocyte or myelocyte stage in their bone marrow (**Fig. 2a**). A synopsis of the clinical features is given in **Table 1**, and further immunological data are presented in **Supplementary Table 1** online.

Qualitative analysis of the genome scan genotypes showed that D1S2635 (located 156.0 Mb from 1pter in build 35 of the human genome) was the only genome scan marker at which all four affected individuals were homozygous and at which the unaffected siblings had a different genotype. After all available individuals were genotyped at D1S2635, the LOD score for that marker was +3.17 at a recombination fraction (θ) of 0. However, this marker was imperfect, because the affected individual in SCN-III was homozygous for an allele different from the disease-associated allele in the other two families.

Fine mapping on chromosome 1 identified six other markers that had perfect segregation within families and were informative enough to give a single-marker LOD score above +2.0 (summed over SCN-I to SCN-III): D1S514 (120.0 Mb, score +2.62), D1S2696 (120.2 Mb, +2.39), D1S3466 (147.0 Mb, +2.78), D1S2624 (153.4 Mb, +3.06), D1S1653 (154.7 Mb, +3.08), and D1S2707 (156.9 Mb, +2.75). Two-marker analysis using D1S3466 and D1S2624 gave a peak LOD score of +3.95 with a nearly flat LOD score curve. Adding a third marker, D1S1653, boosted the peak LOD score to +4.15. For the purpose of identifying positional candidate genes, we defined the minimal critical linkage interval as the interval in which consanguineous families SCN-I and SCN-III have their maximum positive scores at $\theta = 0$, and the three affected individuals therein are homozygous for the same allele. To obtain a maximal interval, we extended by one marker on each side. The minimal interval is from D1S442 (143.1 Mb) through D1S2624 (153.4 Mb), and the maximal

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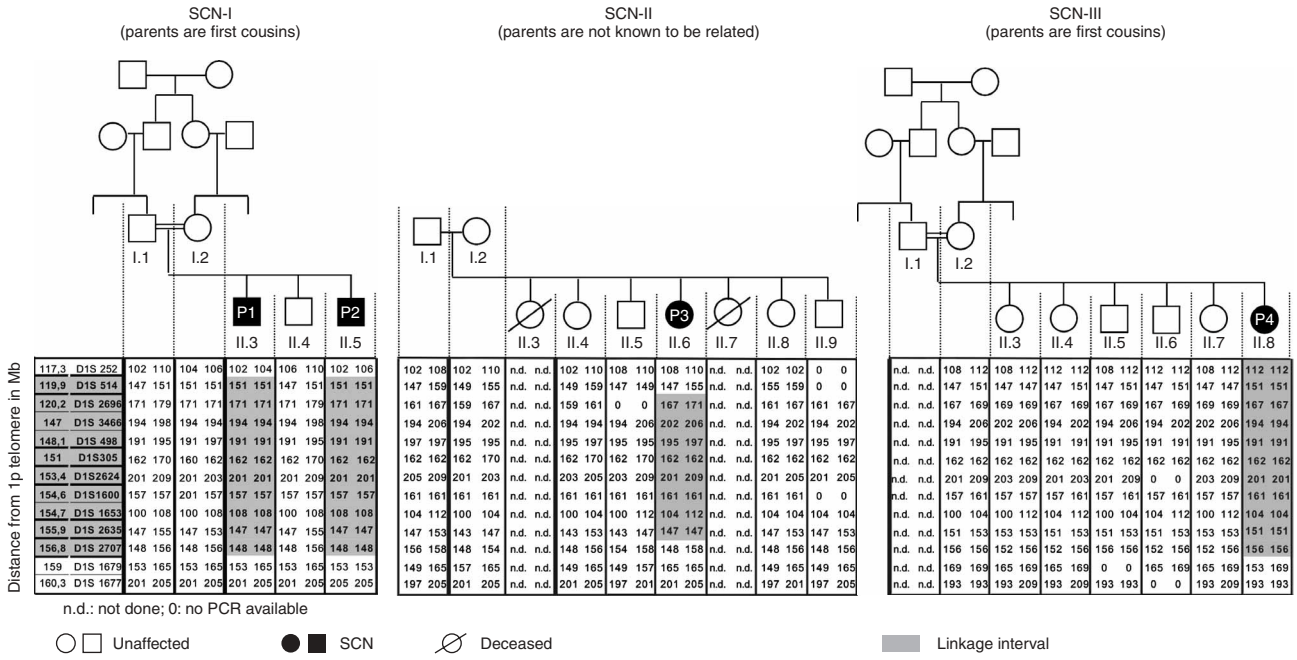

Figure 1 Haplotypes on chromosome 1q. Pedigrees of three unrelated families with severe congenital neutropenia and allele distribution in affected individuals (filled symbols) and healthy family members (open symbols). P1–P4 refer to numbers of individuals in **Table 1**. The homozygous part of the linkage interval within each family is shown in gray. We considered the minimal linkage interval for the study to be the intersection of the intervals for SCN-I and SCN-III, and we concentrated on the subintervals where the affected individuals shared the same allele. We did not use SCN-II to further restrict the overall linkage interval because the lack of documented consanguinity in SCN-II suggested that the affected individual might have two distinct heterozygous mutations.

interval is D1S2696 (120.2 Mb) through D1S1600 (154.6 Mb). In build 35 of the human genome, there are 234 genes or predicted genes in the minimal interval and an additional 41 genes in the maximal interval.

We identified several functional candidate genes among these 275 genes in the maximal interval. Sequencing of genomic DNA from affected individuals showed some wild-type sequences, including *MAPBPIP* (also known as *HSPC003*), *RAB25* and *IL6R*. We considered *HAX1*, localized at 151.1 Mb from 1pter, as a candidate gene for SCN because HAX1 participates in B cell receptor–mediated signal transduction[4], it has the potential to regulate the actin cytoskeleton[5,6] and it is proposed to control apoptosis[9,10]. Increased apoptosis in myeloid progenitor cells has been proposed as a potential mechanism accounting for neutropenia in individuals with SCN[2]. Although intrinsic B cell abnormalities have not previously been reported, defective directed migration and aberrant rearrangement of the cytoskeleton of SCN neutrophils have been described[11]. We sequenced *HAX1* (for detailed conditions, see **Supplementary Table 2** online) and identified a homozygous single-nucleotide insertion (position 130-131insA) leading to a premature stop codon (W44X) in all affected individuals (**Fig. 2b**); their healthy siblings and parents had at least one allele with the wild-type sequence. As a consequence, HAX1 was absent in the cells from affected individuals, as shown by protein blot analysis (**Fig. 2c**). Heterozygous carriers of W44X had no detectable phenotype.

To assess the frequency of *HAX1* mutations within a cohort of sporadic and familial individuals with SCN, we sequenced the gene in 63 additional individuals with SCN associated with a documented myeloid maturation arrest, including 21 individuals with mutations in the gene encoding neutrophil elastase (*ELA2*). Fifteen affected individuals had the same 1-bp insertion as the index families, and one individual had a homozygous single–base pair substitution (256C→T) causing the nonsense change R86X. Three affected individuals from

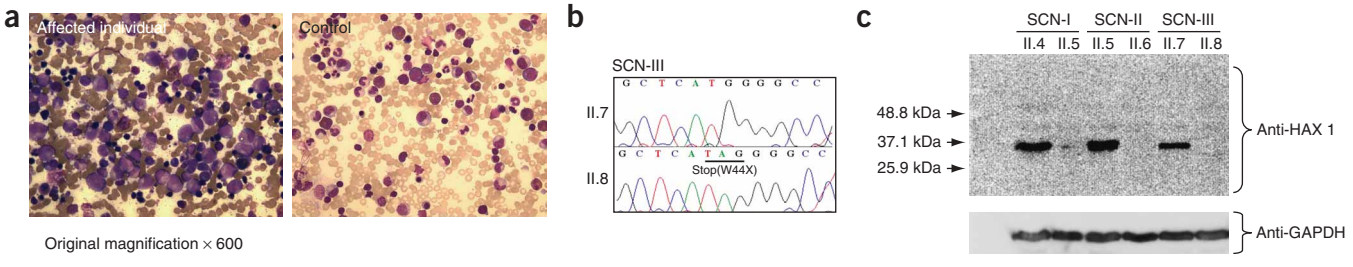

Figure 2 Bone marrow phenotype, HAX1 genotype and HAX1 expression. (**a**) Representative bone marrow phenotype of an individual with SCN (P2) and a healthy individual. Note the characteristic absence of mature neutrophils in the individual with SCN. (**b**) Sequencing of *HAX1* shows a single-nucleotide insertion (A) in exon 2. (**c**) Detection of HAX1 in EBV B cell lines by protein blot analysis (SCNI-II.5 is individual P2, SCNII-II.6 is individual P3 and SCNIII-II.8 is individual P4).

Table 1 Clinical and molecular findings

Individual	Sex	Parental origin	ANC[a]	Bacterial infections	Associated findings	*HAX1*	*ELA2*	*CSFR3*	Therapy[b]	Outcome[c]
1 ('P1')	M	Turkey (K)[d]	224–400	Omphalitis, pneumonia lymphadenitis, sinusitis	β-thalassemia minor, splenomegaly	W44X (G)[e]	WT	WT	G-CSF	Alive, age 11 yrs
2 ('P2')	M	Turkey (K)	192–244	Oral ulcers, otitis, pneumonia, bacteremia	Splenomegaly	W44X (G)	WT	WT	G-CSF	Alive, age 5 yrs
3 ('P3')	F	Turkey (K)	0–410	Pneumonia, skin abscess, stomatitis, tonsillitis	Growth hormone deficiency, splenomegaly	W44X (G)	WT	WT	G-CSF, growth hormone	Alive, age 15 yrs
4 ('P4')	F	Turkey (K)	84–116	Pneumonia, otitis, skin abscess	Tricuspid insufficiency, splenomegaly	W44X (G)	WT	WT	G-CSF	Alive, age 6 yrs
5	M	Turkey (K)	0–464	Lymphadenitis, skin abscess, septicemia, mastoiditis, otitis		W44X (G)	WT	2405C→T (8 yrs after G-CSF)	G-CSF	Alive, age 8 yrs
6	F	Turkey (K)	200	Skin abscess, bronchitis		W44X (G)	WT	WT	G-CSF	Alive, age 6 yrs
7	F	Turkey	535–1,188	Pneumonia, skin abscess, bronchitis	Splenomegaly	W44X	WT	WT	G-CSF	Alive
8	M	Turkey	0–63	Pneumonia, pharyngitis	Splenomegaly, myelodysplasia, extramedullary hematopoiesis	W44X	WT	2423C→T (11 months after G-CSF)[f]	G-CSF, allo-BMT	Alive, age 9 yrs
9	M	Turkey	61	Septicemia, skin abscess		W44X (G)	WT	WT	G-CSF	Alive, age 2 yrs
10	M	Turkey (K)	242	None		W44X (G)	WT	WT	G-CSF	Alive, age 1 yr
11	F	Turkey (K)	0–1,050	Unclassified		W44X (G)	WT	WT	G-CSF	Alive, age 1 yr
12	F	Iran	248	Skin abscess, pneumonia, oral ulcers		W44X	WT	WT	G-CSF	Alive, age 6 yrs
13	M	Iran	608	Omphalitis, skin abscess, oral ulcers, urinary tract infections, pneumonia, otitis		W44X	WT	WT	G-CSF	Alive, age 5 yrs
14	F	Iran	270	Skin abscess, otitis media, pneumonia, oral ulcers	Failure to thrive	R86X	WT	WT	G-CSF	Alive, age 7 yrs
15	M	Turkey	268	Unclassified	Splenomegaly, lymphadenopathy	W44X	WT	WT	G-CSF	Alive, age 14 yrs
16	F	Turkey	200	Gingivitis, pneumonia, otitis		W44X	WT	WT	G-CSF	Alive, age 6 yrs
17	F	Lebanon	0–270	Otitis, enteritis, bronchitis	Splenomegaly	W44X (G)	WT	WT	G-CSF	Alive, age 2 yrs
18	F	Turkey	100–500	Omphalitis, bronchitis		W44X	WT	WT	G-CSF	Alive, age 1 yr
19	M	Lebanon	40–250	Pneumonia, skin abscess, septicemia	46,XY,t(5;9)(q12;p22) in myeloid cells	W44X	WT	2423C→T 2399C→T (13 yrs after G-CSF)	G-CSF	Alive, age 27 yrs
20	F	Turkey	ND	Otitis	Muscular hypotonia	W44X	WT	WT	G-CSF	Alive, age 11 yrs
21	F	Sweden[g]	0–400	Skin abscess, pneumonia, gingivitis, septicemia		Q190X (G)	WT	WT		Died at age 12 yrs
22	F	Sweden[g]	0–270	Otitis, skin abscess, gingivitis, septicemia		Q190X (G)	WT	WT	G-CSF	Alive, age 23 yrs
23	M	Sweden[g]	0–600	Skin abscess, paronychia		Q190X (G)	WT	WT	G-CSF, allo-BMT	Alive, age 22 yrs

Individuals 1,2 (from family SCN-1), 9,10 (siblings) and 21–23 (from the Kostmann family) are the only individuals with an affected relative that we know of. The designations P1, P2, P3 and P4 are used in **Figures 1–4**. BMT, bone marrow transplantation. ND, not done.
[a]ANC: absolute neutrophil count before G-CSF therapy. [b]G-CSF induced increased neutrophil counts in all individuals (required dose, <10 μg/kg body weight). [c]Refers to age in July 2006. [d](K) = Kurdish origin. [e](G) = germline transmission proven by parental heterozygosity. [f]Time after initiation of G-CSF therapy. [g]Individuals from the original Kostmann family (individuals 21, 22 and 23 in our study correspond to patients 1, 4 and 5, respectively, in ref. 13).

the original Kostmann family[12] had the homozygous germline mutation 568C→T (Q190X) (**Supplementary Fig. 1** online), providing definitive proof that Kostmann disease is caused by HAX1 deficiency. None of the individuals with SCN in our cohort was heterozygous for *HAX1* mutations. However, further studies are needed to determine the prevalence of *HAX1* mutations in affected individuals, as our access to SCN samples may have been biased. We screened 200 healthy central European individuals for the presence of the 130-131insA allele and found none. In a healthy Swedish control population ($n = 125$), we determined the allele frequency of the 568C→T mutation to be 1 out of 250 chromosomes. We also sequenced *ELA2*, previously associated with cyclic[13] and congenital[8] neutropenia, in all affected individuals with *HAX1* mutations. Notably, we did not find any affected individuals with mutations in both *ELA2* and *HAX1* (**Table 1**), suggesting that these genes define two mutually exclusive groups of individuals with SCN.

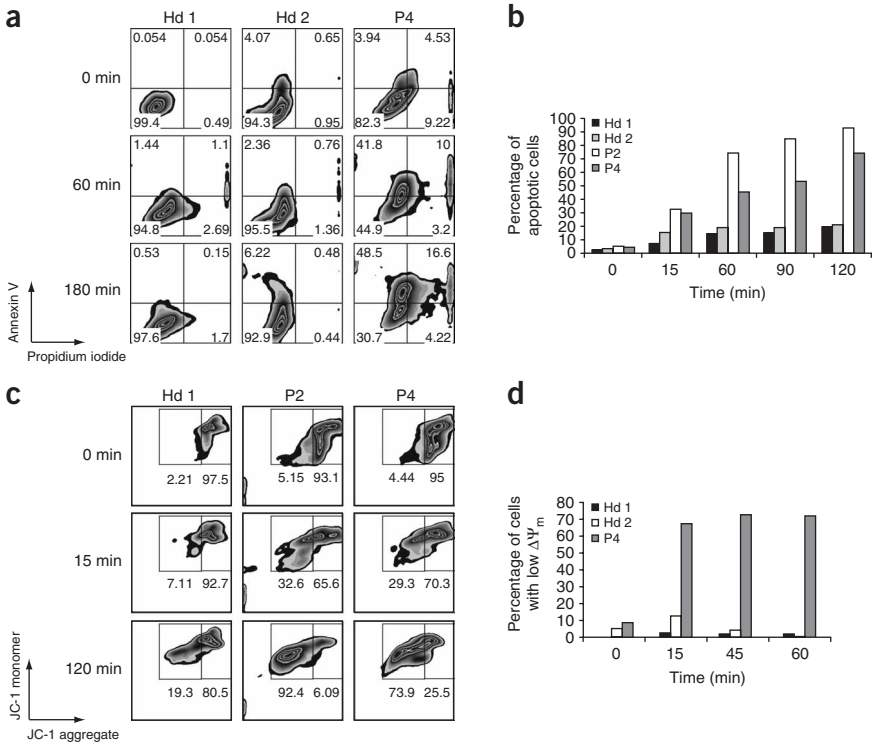

Figure 3 Apoptosis and mitochondrial membrane potential in HAX1-deficient granulocytes. (**a**) FACS plots showing apoptosis of purified neutrophils upon exposure to TNFα. As an additional control, healthy donor 1 (HD1) received G-CSF. (**b**) Rate of apoptosis after treatment of purified neutrophils with H_2O_2. Cells were analyzed by FACS, and the percentage of annexin V–positive, propidium iodide–negative cells was plotted. (**c**) FACS plots showing loss of mitochondrial membrane potential ($\Delta\Psi_m$) upon exposure of purified neutrophils to valinomycin. (**d**) Graphical representation showing progressive loss of $\Delta\Psi_m$ in HAX1-deficient neutrophils upon treatment with valinomycin. All experiments were performed on at least two independent occasions. Similar results were seen in cells from P1 and P3.

SCN is a premalignant condition, as up to 21% of affected individuals develop a clonal proliferative disease leading to myelodysplastic syndrome or overt acute leukemia[14,15], often preceded by mutations in the gene encoding the granulocyte colony stimulating factor (G-CSF) receptor (*CSF3R*)[16]. To determine whether *HAX1* mutations predispose to somatic *CSF3R* mutations, we sequenced *CSF3R* in all affected individuals with documented *HAX1* mutations and reanalyzed the data of the SCN registry[7]. In three HAX1-deficient individuals, we identified somatic mutations in *CSF3R* (**Table 1**). In one of the affected individuals, the onset of a myelodysplastic syndrome led to allogeneic bone marrow transplantation. At this time, it is not clear to what extent the malignant transformation is dependent on the underlying *HAX1* mutation, prolonged exposure to G-CSF or a combination of both factors. Further follow-up studies will be required to estimate the risk posed by HAX1 deficiency with regard to the development of somatic *CSF3R* mutations and myelodysplasia or leukemia.

Mitochondria have been recognized as key regulators of apoptosis in many cell types, including neutrophils[17–19]. Permeabilization of mitochondrial membranes is often a rate-limiting process in apoptotic cell death. Mitochondrial inner membrane permeabilization, manifested as a dissipation of $\Delta\Psi_m$, compromises the vital function of mitochondria and leads to cell death. After this trigger, the outer membrane of mitochondria is permeabilized, leading to release of proteins such as cytochrome c, Smac (also known as DIABLO) and HtrA2 (also known as Omi) from the intermembrane space into the cytosol. Cytochrome c is critical for the formation of the apoptosome, whereas Smac and Omi are negative regulators of inhibitor of apoptosis proteins (IAP) by competing with caspases for IAP binding[20]. The core mitochondrial apoptotic pathway is both executed and regulated by members of the B cell leukemia/lymphoma 2 (BCL2) protein family, which has both antiapoptotic and proapoptotic members and controls cell viability via mitochondrial outer membrane permeabilization[21,22].

In parallel to other pro-survival members of the BCL2 family, such as Mcl-1 and A1 (also known as Bfl-1), HAX1 contains two domains reminiscent of a BH1 and BH2 domain[4] and thus may be involved in controlling apoptosis at the level of the mitochondria. Of note, mice with a targeted deletion of A1-a manifest accelerated neutrophil apoptosis[23]. To directly assess the role of HAX1 in apoptosis, we analyzed the rate of apoptosis in primary neutrophils of HAX1-deficient individuals. We incubated purified neutrophils from affected individuals and healthy donors in the presence of tumor necrosis factor α (TNFα) and analyzed them by FACS for the uptake of propidium iodide and staining with annexin-V. As expected, neutrophils from HAX1-deficient individuals showed a higher amount of both spontaneous and TNFα-induced apoptosis compared with control neutrophils (**Fig. 3a** and **Supplementary Fig. 2** online). We saw similar results when we induced apoptosis by H_2O_2 (**Fig. 3b**) or staurosporine (data not shown). Enhanced neutrophil apoptosis in HAX1-deficient cells was associated with increased cleavage of caspase 3/7 (**Supplementary Fig. 2**). In summary, these findings may explain why treatment with G-CSF, a cytokine with known antiapoptotic functions[24], alleviates the neutropenia phenotype in individuals with SCN.

In view of its preferential mitochondrial localization[4], we reasoned that HAX1 might be involved in stabilizing the mitochondrial membrane potential ($\Delta\Psi_m$) in neutrophils. To visualize $\Delta\Psi_m$, we stained neutrophils from affected individuals and healthy controls with the dual-emission indicator dye 5,5′,6,6′ tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1), which accumulates in mitochondria and forms J-aggregates emitting an orange fluorescence. Upon loss of the mitochondrial membrane potential (for instance, on exposure to the specific K^+ ionophor valinomycin), JC-1 adopts a monomeric conformation and emits green fluorescence. Neutrophils isolated from HAX1-deficient individuals showed a rapid dissipation of $\Delta\Psi_m$, whereas the inner mitochondrial membrane potential in neutrophils from healthy individuals was maintained (**Fig. 3c**,**d**). Similar results were seen in myeloid cells that differentiated *in vitro* (data not shown). These findings are in line with our observation of increased apoptosis in HAX1-deficient neutrophils as well as with the increased release of cytochrome c from these organelles in myeloid progenitor cells[2] and suggest that HAX1 is involved in stabilizing the mitochondrial membrane potential.

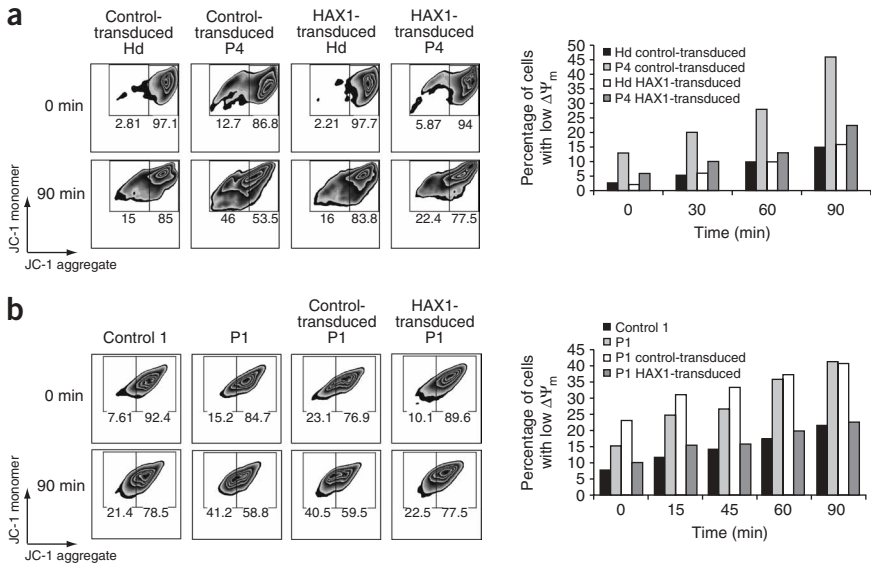

Figure 4 Reconstitution of $\Delta\Psi_m$ in myeloid progenitor cells and fibroblasts after retroviral *HAX1* gene transfer. (**a**) Left: representative FACS plots indicating loss of valinomycin-induced $\Delta\Psi_m$ in myeloid cells that differentiated *in vitro* and reversion of $\Delta\Psi_m$ upon retroviral *HAX1* gene transfer. Right: graphical representation of $\Delta\Psi_m$ reconstitution showing all measured time points. (**b**) Left: representative FACS plots indicating loss of valinomycin-induced $\Delta\Psi_m$ in fibroblasts (P1) and reversion of $\Delta\Psi_m$ upon retroviral *HAX1* gene transfer. Right: graphical representation of $\Delta\Psi_m$ reconstitution in fibroblasts upon retroviral *HAX1* gene transfer, showing all measured time points. We observed similar results in two independent experiments.

As *HAX1* is a ubiquitously expressed gene[4], we were interested to see whether HAX1 deficiency would be associated with altered membrane potential in non-hematopoietic cells. Compared with fibroblasts from healthy donors, HAX1-deficient fibroblasts showed a more rapid loss of their membrane potential when exposed to valinomycin (**Supplementary Fig. 3** online), suggesting that the function of HAX1 in stabilization of the mitochondrial membrane potential may not be limited to neutrophils. Nevertheless, it is mysterious why a seemingly null mutation of a ubiquitously expressed gene causes a myeloid-specific phenotype in individuals with SCN. Perhaps this effect is due to intrinsic differences in the molecular control of apoptosis in neutrophils compared with other cell types. Alternatively, in view of an extremely high cellular turnover rate, neutrophil counts may be particularly sensitive to even slight alterations in the balance of apoptosis.

To unequivocally prove that *HAX1* mutations cause SCN by lowering the threshold for apoptosis upon mitochondrial membrane dissipation, we reconstituted the cellular phenotype of individuals with SCN by retroviral gene transfer. We purified $CD34^+$ cells from affected individuals and healthy controls, transduced them with bicistronic retroviral vectors encoding *HAX1* and a reporter gene (mouse CD24), cultured them *in vitro* until they differentiated into myeloid progenitor cells and analyzed them for maintenance of $\Delta\Psi_m$ upon exposure to valinomycin. As expected, cells transduced with the marker gene showed an accelerated loss of $\Delta\Psi_m$ (**Fig. 4a**). In contrast, affected individuals' myeloid progenitor cells transduced with *HAX1*-expressing virus showed a significantly delayed loss of $\Delta\Psi_m$, similar to wild-type cells that were transduced with a control vector or transduced with HAX1-expressing constructs (**Fig. 4a**). Similarly, maintenance of $\Delta\Psi_m$ was corrected in HAX1-deficient fibroblasts after retroviral gene transfer (**Fig. 4b**).

Our data indicate that HAX1 deficiency causes the phenotype of accelerated loss of $\Delta\Psi_m$ in myeloid cells of individuals with homozygous *HAX1* mutations. Further support for an antiapoptotic function of HAX1 comes from studies analyzing viral proteins. HAX1 interacts with a number of viral proteins, such as the K15 protein of Kaposi's sarcoma–associated herpesvirus[9], Epstein-Barr virus (EBV) nuclear antigen[25], EBV nuclear antigen leader protein (EBNA-LP)[26], and human immunodeficiency virus viral protein R1 (Vpr1) (ref. 27), suggesting that viruses may have developed mechanisms to induce or evade apoptosis via HAX1.

In conclusion, we have shown for the first time the genetic etiology of autosomal recessive SCN and identified a role for the antiapoptotic molecule HAX1 in myeloid cell homeostasis. Thus, our findings point to a mechanism involving mitochondrial control of apoptosis as a regulator of myeloid cell homeostasis in humans. Future genetic studies in individuals with SCN may identify mutations in additional genes controlling the survival of neutrophils. Mutations in *HAX1* should be sought in all individuals with autosomal recessive SCN. We expect that, in the future, *HAX1* mutation status will be used as a variable in large-scale clinical studies as a possible predictor for clinical manifestation, response to treatment, leukemia susceptibility and outcome in individuals with SCN. Our findings may also open up new horizons for clinical and basic research in other premalignant conditions.

METHODS

Participants. Blood, skin, and bone marrow samples were taken upon informed parental consent or participants' consent, according to the guidelines of the local institutional review boards at Hannover Medical School, University of Freiburg and Umeå University Sweden. Participants were referred by pediatric hematologists or identified in our clinic. Central European control samples comprised individuals originating from Germany and Turkey.

Genotyping. A total of 217 markers were genotyped on eight individuals (four affected individuals and four unaffected siblings, including at least one from each family). In the only region selected for fine mapping, an additional 15 markers were genotyped on all available individuals, but one marker was dropped owing to inconsistency, and five other markers were uninformative in at least one family. Reagents for genotyping were purchased from Invitrogen Research Genetics, biomers.net and Qiagen. PCR was performed according to published protocols. PCR products were sequenced on an ABI377 sequencer (PE Applied Biosystems), using the COLLECTION and ANALYSIS software. Allele sizes were determined using the GENOTYPER (PE Applied Biosystems) software.

Genetic linkage analysis. All genotype data were evaluated qualitatively looking for perfect segregation of a marker with the disease and homozygosity in the affected individuals in families SCN-I and SCN-III. The fine-mapping data on chromosome 1 were also evaluated quantitatively by computing LOD scores. These were computed using FASTLINK version 4.1P (refs. 28,29) assuming 0.001 as disease allele frequency and full penetrance. We used equal marker allele frequencies owing to the small sample size. As this study involved multiple families, and the LOD score computations treated each family

separately, there were at least two aspects in which qualitative analysis provided additional information. First, we preferred markers where affected individuals in different families were homozygous for the same allele rather than markers where they were homozygous for different alleles. Second, we preferred markers where the affected individual in SCN-II was homozygous, even though SCN-II does not have known consanguinity. These preferences arose because we suspected that affected individuals in all three families would have the same mutation in the same gene. However, we defined our linkage intervals based only on SCN-I and SCN-III, and we considered the possibility that the affected individual in the non-consanguineous SCN-II family might be a compound heterozygote.

Protein blots. Cell extracts of EBV-immortalized B cell lines were separated by SDS-PAGE, blotted and stained with a monoclonal antibody to HAX1 (BD Biosciences) or antibodies to GAPDH (Santa Cruz). After staining with HRP-conjugated goat antibody to mouse (BD Biosciences), we captured images of chemiluminescence using a Kodak Image Station 440CF.

Assessment of apoptosis and mitochondrial membrane potential. Neutrophils were isolated from peripheral blood; exposed to TNFα (50 ng/ml) (Sigma), H_2O_2 (0.02 M) (Sigma) or staurosporine (5 μM) (Sigma) and analyzed by FACS after staining with annexin-V (Molecular Probes) and propidium iodide (Sigma). Cells were gated on intact neutrophils based on forward scatter and side scatter features. Caspase 3/7 activation was determined by FACS using a commercially available kit (the Vybrant FAM caspase-3 and -7 assay kit (Molecular Probes)). Dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) was determined by FACS after loading the cells with valinomycin (100 nM) (Sigma) and the dye JC-1 (3.5 μM) (Molecular Probes).

Retroviral gene transfer. The human *HAX1* cDNA was cloned into the retroviral vector CMMP[30] containing either GFP or a truncated version of mouse CD24 as a marker gene. Gibbon ape leukemia virus (GALV) envelope pseudotyped retroviruses were generated by tripartite transient transfection of MMP-based transfer vectors together with the envelope plasmid K83.pHCMV-GALVenv and the packaging plasmid pMDgag/pol into the cell line 293T.

$CD34^+$ cells were purified from bone marrow using magnetic microbeads (Miltenyi Biotech). Separation was performed by AutoMACS devices (Miltenyi Biotech). The cells were expanded for 48–72 h in Stemspan SF medium (StemCell Technologies) supplemented with human stem cell factor (100 ng/ml), Flt-3 ligand (100 ng/ml), thrombopoietin (20 ng/ml) and interleukin-6 (20 ng/ml) (PreproTech) and then were transduced by spinoculation on RetroNectin-coated plates. We sorted cells for mouse CD24 expression 48 h later in a FACSAria System (BD Biosciences), and we induced cells to differentiate into myeloid cells using recombinant G-CSF (50 ng/ml) (Amgen) and GM-CSF (50 ng/ml) (Amgen). Functional studies in myeloid cells generated *in vitro* were done after cytokine starvation.

GenBank accession codes. *HAX1* GeneID, 10456; HAX1 protein, NP_006109.2; HAX1 cDNA, NM_006118.3. *ELA2* GeneID, 1991; ELA2 protein, NP_001963.1; *ELA2* cDNA, NM_001972.2. *CSF3R* GeneID, 1441; CSF3R protein, NP_000751.1; *CSF3R* cDNA, NM_000760.2.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

C.K. designed and directed the study; obtained clinical samples; taught and supervised G.A., I.S., K.B., C.R. and G.B.; provided laboratory resources and wrote the manuscript with help from B.G. and A.A.S. The manuscript was then reviewed and approved by all authors. M. Grudzien did the genotyping for linkage analysis and sequenced candidate genes. G.A. performed all gene transfer studies and functional assays on myeloid cells and fibroblasts. M. Germeshausen sequenced *HAX1*, *ELA2* and *CSFR3* and comprehensively analyzed genetic data. I.S. discovered the first *HAX1* mutation and performed sequencing and protein blotting. A.A.S. chose markers to genotype in the linkage region and performed linkage analysis computations. K.B. performed functional immunological assays. C.R. performed functional neutrophil studies and taught G.A. C.Z. cared for patients and collected and curated data in the SCN patient registry. B.S. collected and curated data in the SCN patient registry. N.R. treated patients in Iran and ascertained their samples for this study. G.B. performed functional neutrophil studies and sequenced candidate genes. G.C. and J.-I.H. initiated the Swedish Kostmann family project; G.C. treated the patients, and J.-I.H. and J.P. supervised the project with the support of B.F. N.D. was responsible for Swedish Kostmann gene studies. M.M. sequenced genomic samples from the Kostmann family. B.G. provided laboratory resources, organized patient samples, supervised M. Grudzien and assisted A.A.S. K.W. provided laboratory resources and resources for SCN registry and helped to initiate and carry out the study. M. Grudzien and G.A. contributed equally to this work and are considered *acquo loco*.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Kostmann, R. Infantile genetic agranulocytosis (Agranulocystosis infantilis hereditaria): a new recessive lethal disease in man. *Acta Paediatr.* **45** (Suppl.), 1–78 (1956).
2. Carlsson, G. *et al.* Kostmann syndrome: severe congenital neutropenia associated with defective expression of Bcl-2, constitutive mitochondrial release of cytochrome *c*, and excessive apoptosis of myeloid progenitor cells. *Blood* **103**, 3355–3361 (2004).
3. Cario, G. *et al.* Heterogeneous expression pattern of pro- and anti-apoptotic factors in myeloid progenitor cells of patients with severe congenital neutropenia treated with granulocyte colony-stimulating factor. *Br. J. Haematol.* **129**, 275–278 (2005).
4. Suzuki, Y. *et al.* HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of Src family tyrosine kinases. *J. Immunol.* **158**, 2736–2744 (1997).
5. Gallagher, A.R., Cedzich, A., Gretz, N., Somlo, S. & Witzgall, R. The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton. *Proc. Natl. Acad. Sci. USA* **97**, 4017–4022 (2000).
6. Radhika, V., Onesime, D., Ha, J.H. & Dhanasekaran, N. $G\alpha_{13}$ stimulates cell migration through cortactin-interacting protein Hax-1. *J. Biol. Chem.* **279**, 49406–49413 (2004).
7. Welte, K., Zeidler, C. & Dale, D. Severe congenital neutropenia. *Semin. Hematol.* **43**, 189–195 (2006).
8. Dale, D.C. *et al.* Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. *Blood* **96**, 2317–2322 (2000).
9. Sharp, T.V. *et al.* K15 protein of Kaposi's sarcoma-associated herpesvirus is latently expressed and binds to HAX-1, a protein with antiapoptotic function. *J. Virol.* **76**, 802–816 (2002).
10. Cilenti, L. *et al.* Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death. *J. Biol. Chem.* **279**, 50295–50301 (2004).
11. Elsner, J., Roesler, J., Emmendörffer, A., Lohmann-Matthes, M.L. & Welte, K. Abnormal regulation in the signal transduction in neutrophils from patients with severe congenital neutropenia: relation of impaired mobilization of cytosolic free calcium to altered chemotaxis, superoxide anion generation and F-actin content. *Exp. Hematol.* **21**, 38–46 (1993).
12. Carlsson, G. & Fasth, A. Infantile genetic agranulocytosis, morbus Kostmann: Presentation of six cases from the original "Kostmann family" and a review. *Acta Paediatr.* **90**, 757–764 (2001).
13. Horwitz, M., Benson, K.F., Person, R.E., Aprikyan, A.G. & Dale, D.C. Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. *Nat. Genet.* **23**, 433–436 (1999).
14. Gilman, P.A., Jackson, D.P. & Guild, H.G. Congenital agranulocytosis: prolonged survival and terminal acute leukemia. *Blood* **36**, 576–585 (1970).
15. Rosenberg, P.S. *et al.* The incidence of leukemia and mortality from sepsis in patients with severe congenital neutropenia receiving long-term G-CSF therapy. *Blood* **107**, 4628–4635 (2006).
16. Dong, F. *et al.* Mutations in the gene for the granulocyte colony-stimulating-factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia. *N. Engl. J. Med.* **333**, 487–493 (1995).
17. Green, D.R. & Kroemer, G. The pathophysiology of mitochondrial death. *Science* **305**, 626–629 (2004).

18. Newmeyer, D.D. & Ferguson-Miller, S. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* **112**, 481–490 (2003).
19. Maianski, N.A. *et al.* Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. *Cell Death Differ.* **11**, 143–153 (2004).
20. Kroemer, G. & Reed, J.C. Mitochondrial control of cell death. *Nat. Med.* **6**, 513–519 (2000).
21. Gross, A., McDonnell, J.M. & Korsmeyer, S.J. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* **13**, 1899–1911 (1999).
22. Opferman, J.T. & Korsmeyer, S.J. Apoptosis in the development and maintenance of the immune system. *Nat. Immunol.* **4**, 410–415 (2003).
23. Hamasaki, A. *et al*. Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the *bcl-2*-related A1 gene. *J. Exp. Med.* **188**, 1985–1992 (1998).
24. Maianski, N.A., Mul, F.P.J., van Buul, J.D., Roos, D. & Kuijpers, T.W. Granulocyte colony-stimulating factor inhibits the mitochondria-dependent activation of caspase-3 in neutrophils. *Blood* **99**, 672–679 (2002).
25. Dufva, M., Olsson, M. & Rymo, L. Epstein-Barr virus nuclear antigen 5 interacts with HAX-1, a possible component of the B-cell receptor signalling pathway. *J. Gen. Virol.* **82**, 1581–1587 (2001).
26. Kawaguchi, Y. *et al.* Interaction of Epstein-Barr virus nuclear antigen leader protein (EBNA-LP) with HS1-associated protein X-1: implication of cytoplasmic function of EBNA-LP. *J. Virol.* **74**, 10104–10111 (2000).
27. Yedavalli, V.S. *et al.* Human immunodeficiency virus type 1 Vpr interacts with antiapoptotic mitochondrial protein HAX-1. *J. Virol.* **79**, 13735–13746 (2005).
28. Cottingham, R.W., Jr., Idury, R.M. & Schäffer, A.A. Faster sequential genetic linkage computations. *Am. J. Hum. Genet.* **53**, 252–263 (1993).
29. Schäffer, A.A., Gupta, S.K., Shriram, K. & Cottingham, R.W. Jr. Avoiding recomputation in linkage analysis. *Hum. Hered.* **44**, 225–237 (1994).
30. Klein, C., Bueler, H.R. & Mulligan, R.C. Comparative analysis of genetically modified dendritic cells and tumor cells as therapeutic cancer vaccines. *J. Exp. Med.* **191**, 1699–1708 (2000).